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CCAAT-Enhancer-binding Proteins (C/EBP) Regulate the Tissue Specific Activity of the CD11c Integrin Gene Promoter Through Functional Interactions with Sp1 Proteins*

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The CD11c/CD18 integrin binds lipopolysaccharide, fibrinogen, and heparin, and mediates leukocyte adhesion, spreading, and migration. CD11c/CD18 is primarily found on myeloid cells and its expression is regulated during myeloid differentiation by transcriptional mechanisms acting on the CD11c gene promoter. We now describe that CCAAT/enhancer-binding proteins (C/EBP) contribute to the basal, tissue-specific and developmentally regulated activity of the CD11c promoter. A C/EBP-binding site within the CD11c promoter (CEBP-80) is bound by CEBPα in undifferentiated U937 cells and by C/EBPα- and C/EBPβ-containing dimers in phorbol 12-myristate 13-acetate-differentiating cells, and its disruption decreased the CD11c promoter activity in a cell type-dependent manner. C/EBPα transactivated the CD11c promoter through the CEBP-80 element, and C/EBPα transactivation was also dependent on the Sp1–70- and Sp1–120 Sp1-binding sites. The −90/−50 fragment from the CD11c promoter, containing the adjacent CEBP-80, Sp1–70, and AP1–60 sites, differentially enhanced the activity of the minimal prolactin promoter in hematopoietic and epithelial cells. Altogether, these results demonstrate that C/EBP factors participate in the tissue-restricted and regulated expression of the CD11c/CD18 integrin through functional interactions with Sp1, suggest that Sp1-related factors modulate C/EBPα transcriptional activity on the CD11c promoter, and demonstrate the existence of a composite regulatory element recognized by C/EBP, Sp1, and AP-1 factors and whose enhancing effects are cell-type dependent.

The CD11c/CD18 (p150, 95, CR4, LeuM5) heterodimer of the leukocyte integrin subfamily (1) mediates leukocyte adhesion during immune and inflammatory responses, is a specific receptor for LPS, iC3b, fibrinogen, and heparin, and participates in leukocyte adhesion to and spreading on protein-coated surfaces (1–14). CD11c/CD18 is primarily expressed on myeloid cells (15, 16), although can be induced upon B cell activation and long-term T cell activation (8, 17–19). CD11c/CD18 constitutes one of the best cell surface markers for mononuclear phagocytes (15) and is a diagnostic marker for B cell malignancies such as hairy cell leukemia and chronic lymphocytic leukemia (20, 21).

CD11c/CD18 expression is regulated during myeloid differentiation by mechanisms acting at the level of CD11c gene transcription (16). Determination of the activity of the CD11c gene promoter in distinct cell types has evidenced the importance of the Sp1-binding sites Sp1–70 and Sp1–120 for the basal and myeloid-specific transcription of the CD11c gene (22–24), and demonstrated the essential role that members of the AP-1 family play in that the regulated expression of CD11c during myeloid differentiation (24–26). Furthermore, AP-1 and Sp1 family members appear to have a synergistic effect on the activity of the CD11c promoter through their binding to adjacent *cis*-acting elements (24).

CCAAT/enhancer-binding protein (C/EBP)[1] family members are basic-leucine-zipper transcription factors which recognize specific DNA sequences as either homodimers or heterodimers (27). The C/EBP family includes, at least, six members (C/EBPα, β, γ, δ, ϵ, and CHOP-10/GADD153) which dimerize in a tissue-specific manner, and with highly homologous dimerization and DNA contact domains, and similar DNA binding activities. Members of the C/EBP family have been implicated in regulating the differentiation of distinct mammalian cells, including adipocytes, hepatocytes, and myelomonocytes (27). In fact, C/EBPα, β, and δ expression within the hematopoietic system is restricted to myeloid cells. Based on these facts, and considering the preferential expression of the CD11c/CD18 integrin in differentiated myeloid cells, we have analyzed the role of C/EBP factors in the expression of the CD11c integrin gene in myeloid and other cell types. In the present report we describe the structural and functional characterization of a C/EBP-binding site (CEBP-80) within the CD11c promoter whose occupancy is regulated in a cell type- and differentiation-dependent manner and whose disruption preferentially affects the activity of the CD11c promoter in myeloid cells. The positive regulatory effect of C/EBPα on the activity of the CD11c promoter is dependent on adjacent *cis*-acting elements (Sp1–120, Sp1–70, AP-60). Our results demonstrate the contribution of the C/EBP transcription factors to the tissue-restricted and differentiation-regulated expression of the CD11c/CD18 integrin, reveal a functional interplay among C/EBP, Sp1, and AP-1 family members and identify a cell type-dependent enhancer-like element within the proximal regulatory region of the CD11c promoter.

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[1] The abbreviations used are: C/EBP, CCAAT/enhancer-binding protein; CHOP, C/EBP homologous protein; ECM, extracellular matrix; GADD153, growth arrest and DNA damage inducible gene 153; PMA, phorbol myristate acetate.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfections—The human cell lines HeLa (epithelial carcinoma), JY (lymphoblastoid B), U937 (histiocytic lymphoma), HL-60 (myelomonocytic leukemia), THP-1 (acute monocytic leukemia), and HepG2 (hepatoma), as well as the murine RAW 264.7 macrophage cell line, were cultured in RPMI supplemented with 10% fetal calf serum, 25 mM HEPES, 2 mM glutamine, and 50 μg/ml gentamicin (complete medium), at 37 °C in a humidified atmosphere with 5% CO_2. Induction of differentiation of U937 and HL-60 was carried out with PMA at 5 ng/ml for 48 h (HL-60) or 10 ng/ml for 24 h (U937) and at a density of 5×10^5 cells/ml (16, 26). Spleen-derived hairy cells from Hairy Cell Leukemia patients (90% CD19 positive cells, 99% CD11c positive cells) were kindly provided by Dr. H. C. Kluin-Nelemans (University Medical Center, Leiden, The Netherlands). Peripheral blood monocytes and B lymphocytes were isolated according to standard procedures and activated with PMA as described (8). The *Drosophila* Schneider cell line SL2 was cultured in Schneider's medium supplemented with 15% fetal calf serum, 2 mM glutamine, and 50 μg/ml gentamicin, and grown in 100-mm culture plates at a density of 20×10^6/plate.

U937, HL-60, HeLa, and JY cells were transfected by electroporation or using Lipofectin, as described previously (22, 23, 28). RAW 264.7 and HepG2 were transfected using dioleoyloxypropiltrimethylammonium methylsulfate following the manufacturer's instructions (Boehringer Mannheim). PMA treatment was always carried out 16–20 h before luciferase determinations. The amount of DNA in each transfection was normalized by using carrier DNA. For comparative purposes between cell lines and reporter gene constructs, transfection efficiencies were normalized by cotransfection with the β-galactosidase expression plasmid pCMV-βgal. The activity of each reporter construct was expressed relative to the activity produced by the wild type reporter construct (pCD11C160-Luc) in each cell line (relative promoter activity). Transactivation experiments in SL2 cells were carried out with Lipofectin, as described previously (23), and using the Sp1 expression plasmid pPacSp1 (generously provided by Dr. Robert Tjian, University of California at Berkeley, CA). Transactivation experiments in HeLa cells used 0.5 μg of the corresponding reporter plasmid and 5 μg of the distinct expression vectors for C/EBPα (MSV-EBPα), C/EBPβ (MSV-EBPβ), or C/EBPδ (MSV-EBPδ), which were kindly provided by Dr. S. McKnight (Tullarik Inc., South San Francisco, CA). To measure the transactivation activity of each plasmid, promoter activity induction was defined as the ratio of relative light units in cells transfected with expression vector to the relative light units produced by cells transfected with an equimolar amount of insertless vector (pEMBL19 including the MSV promoter), after background substraction and normalizing for cell number (25).

Plasmids and Site-directed Mutagenesis—The CD11a- and CD11c-based reporter constructs pCD11A170-Luc, pCD11A100-Luc, and pCD11C160-Luc have been previously described (23, 28). The mutant reporter plasmids pCD11C160(−70mut)-Luc, pCD11C160(−120mut)-Luc, pCD11C160(−60mut)-Luc, and pCD11C160(−85mut)-Luc, containing mutations at the Sp1–70, Sp1–120, AP1–60, and Myb-85 sites, have been also reported (23, 25, 26). The constructs pCD11C160(−5mut)-Luc and pCD11C160(−10mut)-Luc, harboring mutations at the PU1–5 PU.1-binding site and the GABP-10 GABP-binding site, will be described elsewhere.[2] Construction of the pCD11C160(−80mut)-Luc plasmid, harboring mutations at CEBP-80 which disrupt C/EBP-binding, was accomplished by a double polymerase chain reaction procedure on the CD11c promoter insert in pCD11C160-Luc, using oligonucleotides pXP2–160 (5′-CTTGGATCCAAGCCAAGTCATCTGATGAGAG-3′) (25, 26), and oligo Box Dmut4 antisense (5′-CCTCGGATCAGGACTAGTCTCTGC-3′) for the upstream polymerase chain reaction, and oligonucleotides Box D mut4 sense and oligonucleotide CD11c pXP2 +43 (5′-GATCTCGAGCTCCTGGGCCG-3′) (25) for the downstream polymerase chain reaction fragment. Both polymerase chain reaction fragments were digested with *Bam*HI/*Spe*I and *Spe*I/*Xho*I, respectively, and ligated into *Bam*HI/*Xho*I-digested pXP2. All mutations and constructs were confirmed by DNA sequencing.

To evaluate the influence of the fragment containing the CEBP-80, Sp1–70, and AP1–60 sites on an heterologous promoter, the double-stranded oligonucleotide CESpAP (5′-GATCGTCGACGATCAGTTGCGTACTCTGCCCGCCCCCTCTGACTCATGCTCTAGACTCGAGGCAT-3′) was synthesized, spanning nucleotides −90/−50 from the CD11c promoter and including a *Sal*I site at the 5′-end and a *Xho*I site at the 3′-end. The CESpAP sequence was placed upstream of the rat minimal prolactin promoter/luciferase cDNA unit within the pRL-Luc plasmid, and both in the sense and antisense orientations. To produce dimers of the −90/−50 fragment arranged in a head-to-tail orientation, the CESpAP oligonucleotide was self-ligated and the resulting product subjected to digested with *Sal*I and *Xho*I. The *Xho*I- and *Sal*I-resistant dimers were isolated and purified by acrylamide gel electrophoresis and subsequently cloned into *Xho*I-digested pRL-Luc. All constructs were confirmed by DNA sequencing.

Electrophoretic Mobility Shift Assays (EMSA)—EMSA was performed basically as described (26). Briefly, 50 ng of the corresponding CD11c promoter-based probes were labeled using avian myeloblastosis virus reverse transcriptase (7 units) and 50 μCi of [^{32}P]dCTP to an specific activity of approximately 10^8 cpm/μg. After incubation of 0.5 ng of probe with 2–5 μg of nuclear extract for 20 min at 4 °C, 12 μl of each reaction was separated by electrophoresis at 15 V/cm and 4 °C on 5% polyacrylamide gels. For inhibition assays, unlabeled competitor oligonucleotides (100-fold molar excess) and polyclonal antisera were preincubated with the nuclear extracts at 4 °C for 30 min before the addition of the probe. Rabbit antisera against C/EBPα, C/EBPβ, and C/EBPδ were obtained from Santa Cruz Biotechnology (anti-C/EBPα), and kindly provided by Dr. V. Poli (anti-C/EBPβ, Istituto de Ricerche di Biologia Molecolare, Rome, Italy), Dr. S. McKnight (anti-C/EBPα, anti-C/EBPβ, and anti-C/EBPδ, Tullarik Inc., South San Francisco, CA), and Dr. U. Schibler (anti-C/EBPβ, Université de Géneve, Switzerland). Nuclear extract preparation was done as described (23, 26, 29).

The CD11c promoter-based oligonucleotide Box D used for EMSA was (−94) 5′-CCTCGGATCAGTTGCGTACTCTGCC-3′ (−70), while those used for competition experiments included CEBP-CONS (consensus binding site for C/EBP proteins), CEBP-CONSmut, 2xMyb (containing two Myb-binding sites)(30), E4TF1 (including a GABP/E4TF1-binding site) (31), and Box A, a CD11c-derived oligonucleotide including promoter sequences −19/−3 (5′-TCTGCCCACTTGCTTCC-3′) that contains the E-box sequence CACTTG. For determination of the nucleotides implicated in C/EBP binding, oligonucleotides including distinct mutations on the sequences surrounding CEBP-80 were used, and their relative positions and mutations are shown in Fig. 1*A*.

RESULTS

Identification of a C/EBP-binding Site within the CD11c Gene Promoter—We have previously demonstrated that recombinant c-Myb binds the Myb-85 element within the CD11c promoter (25). Since Myb-85 (−86 CAGTTGC −80) overlaps an E-box sequence (−86 **CANNTG −81**) and a sequence closely conforming to the consensus C/EBP-binding site (32) (−84 GTTGCGTA −77) (Fig. 1*A*), an oligonucleotide spanning from −94 to −70 (Box D) was subjected to EMSA to determine the pattern of protein binding to this region of the CD11c promoter. As shown in Fig. 1*B*, myeloid U937 nuclear extracts produced specific retarded complexes on Box D (marked CEBP) whose formation was prevented by a 100-fold molar excess of cold oligonucleotide probe and whose intensity and mobility differed among cell types (Fig. 2 and data not shown).

To determine whether the formation of the retarded species was dependent on either E-box or Myb-binding site, inhibitory experiments were performed with consensus and mutated oligonucleotides (Fig. 1*B*). The specific complexes were not inhibited by a 100-fold molar excess of a Myb-binding site (25, 30), by an oligonucleotide from the CD11c promoter containing a distinct E-box (CACTTG) or by an additional unrelated sequence containing a GABP(E4TF1)-binding site (31)(Fig. 1). On the other hand, an oligonucleotide containing a consensus C/EBP-binding site (CEBP-CONS) completely prevented the formation of the retarded species, while CEBP-CONSmut, where the consensus C/EBP site is disrupted, had no effect on complex formation (Fig. 1*B*). In addition, while mutations at positions flanking the putative C/EBP-binding site abolished complex formation (Box Dmut2, Box Dmut 3), mutations that completely prevent c-Myb binding and eliminate the E-box sequence (Box Dmut1)(25) only partially affected CEBP complex formation (Fig. 1, *A* and *B*). By contrast, Box Dmut4 oligonucleotide did not inhibit complex formation (Fig. 1). Altogether,

[2] C. López-Rodríguez and A. L. Corbí, manuscript in preparation.

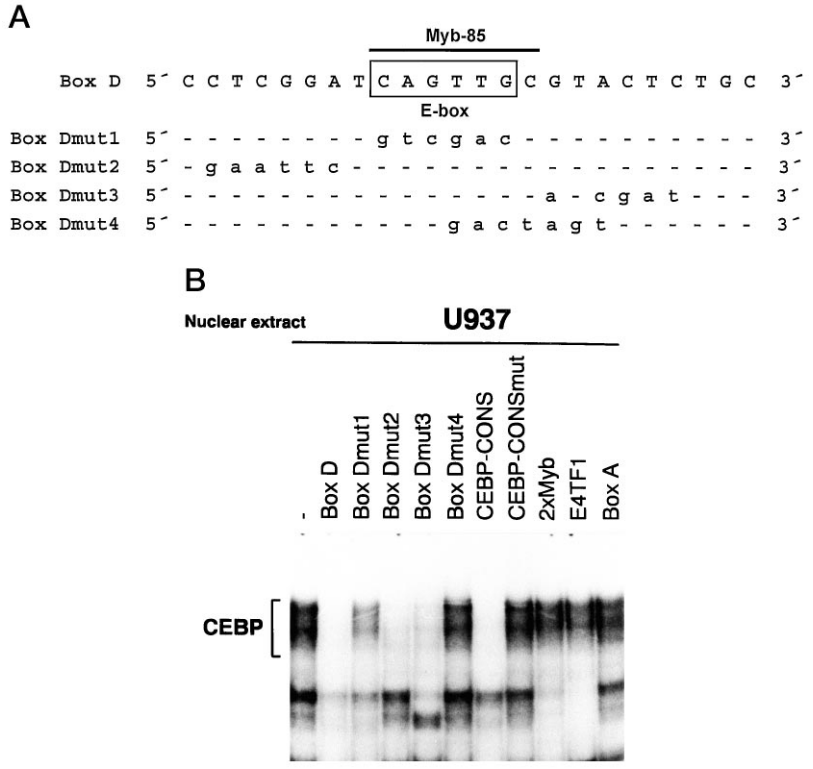

FIG. 1. **Specificity of the EMSA complexes formed on the Box D oligonucleotide.** *A*, sequence of the Box D oligonucleotide probe and the Box Dmut series. Mutated nucleotides are shown in *lowercase*. The position of the E-box and the Myb-binding site are indicated. *B,* nuclear extracts from U937 myeloid cells were tested for their capacity to recognize an oligonucleotide surrounding the −80 position of the CD11c promoter (Box D). The specificity of the retarded species was determined in the presence of a 100-fold molar excess of the indicated oligonucleotides which included the cold Box D oligonucleotide, four distinct Box D-derived mutants (Box Dmut1–4), a consensus C/EBP-binding site (CEBP-CONS), and its corresponding mutant (CEBP-CONSmut), a CD11c promoter-derived oligonucleotide including the −19/−3 fragment (Box A), and oligonucleotides containing two Myb-binding sites (2xMyb)(30) or a GABP/E4TF1-binding site (E4TF1) (31). The specific retarded complexes are indicated by *CEBP*.

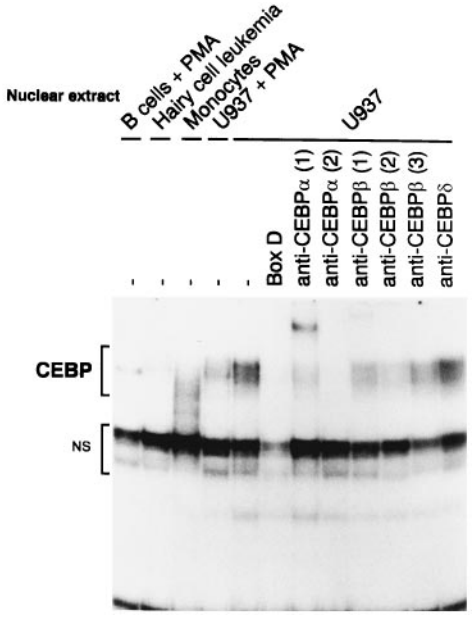

FIG. 2. **Identification of C/EBP factors in the retarded species on Box D (CEBP-80).** Nuclear extracts from activated B cells, hairy cell leukemia cells, monocytes, and untreated or PMA-treated U937 cells were tested for their ability to recognize the Box D oligonucleotide, either in the absence (−) or in the presence of cold Box D oligonucleotide (*lane Box D*) or distinct polyclonal antibodies against C/EBP (*1*, from Santa Cruz; *2*, from Dr. S. McKnight), C/EBPβ (*1*, from Dr. S. McKnight; *2*, from Dr. V. Poli; *3*, from Dr. U. Schibler), or C/EBPδ (from Dr. S. McKnight). The specific retarded complexes are indicated by *CEBP*.

these results indicated that the sequence −83 TTGCGTA −77 (hereafter termed CEBP-80) overlaps the Myb-85 element but is bound by proteins with a DNA-binding specificity distinct from c-Myb. Instead, the CEBP-80 element is bound by factors which recognize high affinity C/EBP-binding sites and require an intact TTGCGTA sequence.

The identity of the involved factors was unambiguously determined through the use of polyclonal antisera against members of the C/EBP protein family. As shown in Fig. 2, the specific interactions in U937 were either supershifted or completely prevented by polyclonal antisera against C/EBPα, while certain anti-CEBPβ antisera weakly affected complex formation and anti-C/EBPδ antiserum had no effect. Therefore, the CD11c promoter CEBP-80 element is specifically recognized by transcription factors of the C/EBP family which, in the case of undifferentiated U937 cells, are predominantly C/EBPα homodimers and with a minor proportion of C/EBPβ-containing dimers (Fig. 2). The pattern of C/EBP-80-bound proteins differed among cell types, even within the same cell lineage (Figs. 2 and 3, and data not shown). Nuclear extracts from activated B cells and hairy cell leukemia cells exhibited a single and faint retarded complex, while those seen in monocytes were more heterogeneous and with a higher mobility (Fig. 2). Within the myeloid lineage, the level of CEBP-80-bound complexes, the relative proportion of C/EBPβ and C/EBPα, and the changes in CEBP80-bound factors associated with myeloid differentiation also differed among the HL-60, U937, and THP-1 cell lines, with C/EBPδ-containing complexes only seen in THP-1 cells (Fig. 3). Monocytic differentiation of HL-60 cells greatly increased CEBP-80-bound species while PMA-mediated differentiation of U937 dramatically reduced the levels of CEBP-80-bound C/EBP factors (Fig. 2). Kinetic studies revealed the appearance of C/EBPβ- and C/EBPδ-containing dimers along U937 differentiation and indicated that CEBP-80-bound factors are undetectable 48 h after PMA addition in U937 cells (Fig. 4). As a whole, the structural analysis revealed that C/EBPα-, C/EBPβ-, and C/EBPδ-containing dimers bind specifically to the CEBP-80 element in the CD11c gene promoter and in a cell type- and differentiation-dependent manner.

Functional Relevance of the CEBP-80 Element—The differential occupancy of the CEBP-80 element suggested that it might contribute to the tissue specific activity of the CD11c promoter. The functional effect of mutations abolishing C/EBP binding to CEBP-80 were determined in transient transfection assays and indicated that CEBP-80 disruption greatly de-

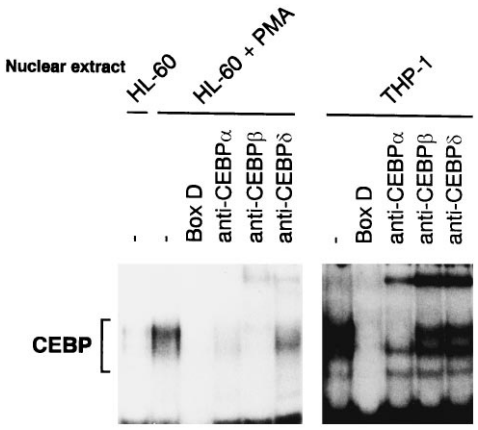

FIG. 3. **Identification of C/EBP factors bound to CEBP-80 in myeloid cell lines.** EMSA was performed on the Box D oligonucleotide using nuclear extracts from HL-60, PMA-differentiated HL-60, and THP-1 myeloid cells, and either in the absence (−) or in the presence of competitor oligonucleotides (*Box D*) or polyclonal antisera against C/EBPα, β, or δ (from Dr. S. McKnight). The specific retarded species are denoted by *CEBP*.

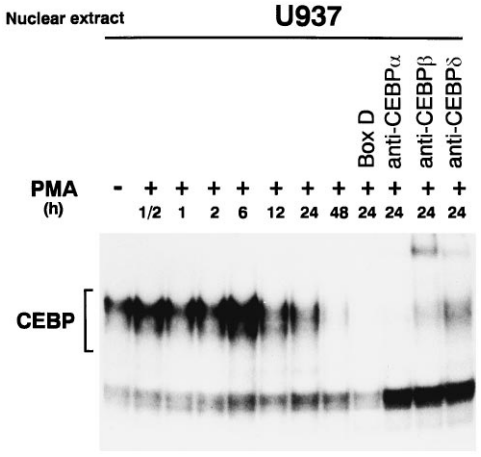

FIG. 4. **Pattern of C/EBP binding to CEBP-80 along PMA-triggered U937 monocytic differentiation.** EMSA was performed on the Box D oligonucleotide using nuclear extracts from U937 cells either untreated (−) or treated with PMA for the indicated times, and either in the absence or presence of competitor oligonucleotides (Box D) or polyclonal antisera against C/EBPα, β, or δ (kindly provided by Dr. S. McKnight). The specific retarded species are denoted by *CEBP*.

creased the CD11c promoter activity in all the human myeloid cell lines tested (Fig. 5). The CD11c promoter activity dropped to 11% in HL-60 cells, 16% in U937 cells, and 20% in the more differentiated mouse macrophage RAW 264.7 cell line (Fig. 5). Mutation of CEBP-80 had a lower effect in other cell types as the promoter activity decreased to 40% in the JY lymphoblastoid cell line, to 46% in the epithelial carcinoma HeLa cell line, and to 60% in HepG2 hepatoma cells (Fig. 5). Therefore, disruption of the CEBP-80 element caused a cell type-dependent decrease in the activity of the CD11c promoter, indicating that C/EBP binding to CEBP-80 might directly contribute to the tissue-restricted expression of the CD11c/CD18 integrin.

The constitutive expression of C/EBPα in undifferentiated myeloid cells (33) suggested that C/EBPα might be responsible for most of the positive regulatory effect of CEBP-80 on the activity of the CD11c promoter and, consequently, transactivation experiments were performed in HeLa cells. Expression of C/EBPα significantly augmented the activity of the CD11c promoter (2-fold increase), an effect which was absolutely dependent on the integrity of the CEBP-80 element (Fig. 6). On the other hand, transfection of expression plasmids for C/EBPβ or C/EBPδ under similar conditions had no effect on the activity of the CD11c promoter (Fig. 6), while the activity of the CD11a promoter increased upon transfection of C/EBPα, C/EBPβ, or C/EBPδ (Fig. 6). Therefore, C/EBPα contributes to the activity of the CD11c promoter through recognition of the CEBP-80 element.

Functional Interplay between C/EBP and Sp1 Family Factors on the CD11c Promoter—C/EBP and Sp1 factors can recognize their respective binding sites within the CD11c promoter independently of one another (Ref. 23 and this paper): C/EBP factors bind the Box D oligonucleotide, which does not include any Sp1-binding site, and Sp1 interacts with Sp1–70 or Sp1–120 in the absence of any C/EBP-binding site (23). However, the Sp1 contribution to the tissue specific activity of the CD11c promoter (23), the cell type-dependent influence of CEBP-80 on the CD11c promoter activity, and the proximity of the Sp1–70, Sp1–120, and CEBP-80 elements prompted us to analyze whether C/EBP and Sp1 factors were functionally collaborating for the tissue-restricted expression of CD11c. The effect of CEBP-80 disruption on the Sp1 transcriptional activity was evaluated in transactivation experiments in *Drosophila* SL2 cells, which are devoid of Sp1, and revealed that mutation of CEBP-80 led to a consistent increase in the CD11c promoter transactivation by Sp1 (2–3-fold), indicating that occupancy of CEBP-80 influences the positive transcriptional activity of Sp1 on the CD11c promoter (data not shown). To determine whether integrity of Sp1-binding sites is required for the C/EBPα transcriptional activity on the CD11c promoter, the inverse set of experiments was performed. As expected, C/EBPα transactivation totally depended on CEBP-80 (Fig. 7). However, mutation of either Sp1–70 or Sp1–120 completely abolished the capacity of C/EBPα to transactivate the CD11c promoter (Fig. 7), thus demonstrating that C/EBPα binding is required but is not sufficient for transactivation of the CD11c promoter and indicating that the positive transcriptional effect of C/EBPα on the CD11c promoter is dependent on the integrity of the adjacent Sp1-binding sites Sp1–70 and Sp1–120. Furthermore, mutation of the adjacent AP1–60 also partially inhibited the C/EBPα transactivation (Fig. 7), in agreement with the reported AP-1/Sp1 collaboration on the proximal CD11c promoter (24). By contrast, elimination of the binding sites for Myb, PU.1, or GABP had no effect on the ability of C/EBPα to transcriptionally activate the CD11c promoter (Fig. 7). Altogether, these results demonstrate that the positive regulatory activity of C/EBPα is dependent on the adjacent Sp1–70, Sp1–120, and AP1–60 sites within the CD11c promoter. Recent studies on the rat *CYP2D5* gene have shown that Sp1 proteins synergize with C/EBPβ at the transcriptional level and facilitate their recognition of DNA elements greatly differing from canonical C/EBP-binding sites (34, 35). In fact, C/EBPβ was not capable of stably interacting with the *CYP2D5* cryptic C/EBP-binding site unless Sp1 was bound at a closely juxtaposed site (34, 35). This does not appear to be the case in the CD11c promoter as C/EBP proteins can recognize the CEBP-80 element in the absence of the adjacent Sp1–70 or Sp1–120 site (Figs. 1–4), and anti-Sp1 antibodies or Sp1 consensus oligonucleotides do not affect CEBP-80 recognition (data not shown).

The −90/−50 Fragment of the CD11c Promoter Functions as an Enhancer on an Heterologous Promoter—Our results, when considered in conjunction with the reported functional interaction between factors bound at Sp1–70 and AP1–60 (24), suggest that the CEBP-80, Sp1–70, and AP1–60 elements might constitute a functional unit within the CD11c gene promoter whose functional interplay would represent the basis for the tissue-specific and differentiation-regulated expression of the CD11c integrin gene. To determine whether the fragment en-

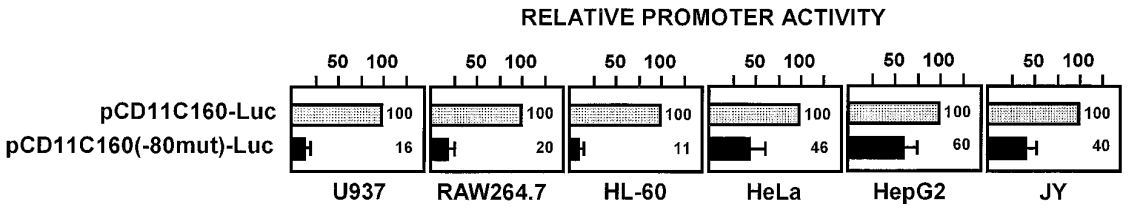

FIG. 5. **Contribution of the CEBP-80 element to the activity of the CD11c promoter.** U937, HL-60, RAW264.7, JY, HeLa, and HepG2 cells were transfected with the indicated reporter constructs and the luciferase activity determined and expressed relative to the activity produced by the wild type reporter construct (pCD11C160-Luc) in each cell line (relative promoter activity). The average of three to four independent experiments, with distinct DNA preparations, is shown, and *bars* indicate standard deviations.

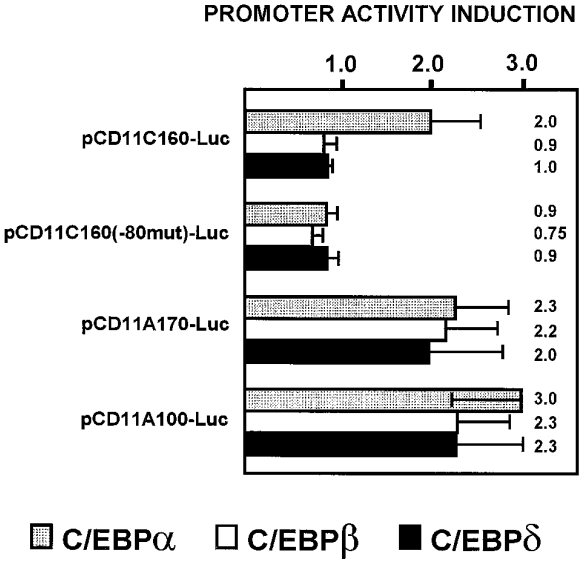

FIG. 6. **Transactivation of the CD11c promoter by C/EBPα.** Human epithelial carcinoma HeLa cells were transfected with the pCD11c160-Luc, pCD11C160(−80mut)-Luc, pCD11A170-Luc, or pCD-11A100-Luc reporter constructs and in the presence of either expression vector for C/EBPα, C/EBPβ, or C/EBPδ or an insertless vector. Promoter activity induction represents the activity of each reporter construct when cotransfected with the corresponding C/EBP expression vector and relative to the activity of the same construct cotransfected with an insertless vector containing the same promoter. Each transfection was performed at least three times, using distinct DNA preparations, and the mean and standard deviations are indicated.

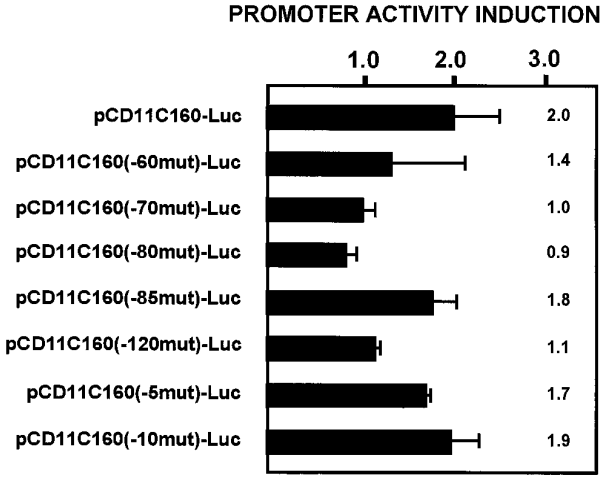

FIG. 7. **Dependence of the C/EBPα transcriptional activity on adjacent transcription factor-binding sites.** The CD11c promoter derived constructs pCD11C160-Luc (wild type), pCD11C(−5mut)-Luc (mutated at PU1–5), pCD11C(−10mut)-Luc (mutated at GABP-10), pCD11C160(−60mut)-Luc (mutated at AP1–60), pCD11C160(−70mut)-Luc (mutated at Sp1–70), pCD11C160(−80mut)-Luc (mutated at CEBP-80), pCD11C(−85mut)-Luc (mutated at Myb-85), and pCD11C(−120mut)-Luc (mutated at Sp1–120), were transfected in HeLa cells with either a C/EBPα expression vector (MSV-CEBPα) or the corresponding insertless vector. Promoter activity induction represents the activity of each reporter construct when cotransfected with the MSV-CEBPα vector and relative to the activity of the same construct cotransfected with the empty vector. Each transfection was performed at least three times using distinct DNA preparations and the mean and standard deviations are shown.

compassing the CEBP-80/Sp1–70/AP1–60 sites could confer transcriptional activation when isolated from the flanking sequences, the −90/−50 region of the CD11c promoter was linked to an heterologous promoter in the sense and antisense orientations, and either as a monomer or a head-to-tail dimer. In myeloid U937 cells, the presence of the −90/−50 region greatly increased the activity of the rat minimal prolactin promoter either in the sense (67-fold) or antisense (147-fold) orientation (Fig. 8). The presence of an additional fragment doubled the enhancing effect in the sense orientation, but provided no additional increase to the enhancing effect of the fragment in the antisense orientation (Fig. 8). The enhancing effect was also observed in epithelial HeLa cells, where the activity of the rat prolactin minimal promoter was increased either 228 times (sense) or 99 times (antisense CESpAP) (Fig. 8). Unlike in the case of U937 cells, dimerization of the antisense CESpAP oligonucleotide produced a higher enhancing effect than the monomeric antisense fragment (222- *versus* 99-fold). Therefore, the −90/−50 region from the CD11c promoter is capable of greatly potentiating the transcription from an heterologous TATA-containing promoter, independently on its relative orientation, thus indicating that it acts as an enhancer. Furthermore, the differential potentiating effects of the CESpAP oligonucleotide in U937 and HeLa cells indicate that the enhancer activity of the −90/−50 region from the CD11c promoter is cell type-dependent, probably reflecting its recognition by members of the C/EBP and AP-1 transcription factor families.

DISCUSSION

We present evidence that C/EBP transcription factors modulate the basal and tissue specific activity of the CD11c integrin gene promoter by recognition of the CEBP-80 element and through functional cooperation with factors interacting with the Sp1–70 and Sp1–120 *cis*-acting elements. CEBP-80-mediated C/EBPα transactivation of the CD11c promoter is absolutely dependent on the integrity of the Sp1–70 and Sp1–120 Sp1-binding sites, implying that the functional interplay of Sp1-related proteins and C/EBP factors is an important parameter for CD11c/CD18 integrin expression and explaining the involvement of the Sp1–70 and Sp1–120 elements in the tissue specific activity of the CD11c promoter (23). Transcriptional synergy between C/EBP and Sp1 proteins has only been shown on the rat cytochrome CYP2D5 gene (34, 35), where Sp1 also facilitates C/EBPβ binding to a very weak C/EBP-binding site that is not recognized by C/EBPβ unless a functional Sp1-binding site is closely juxtaposed (34, 35). Unlike in the case of the *CYP2D5* gene, C/EBP factors do recognize the CEBP-80 element in the absence of both Sp1–70 and Sp1–120 elements,

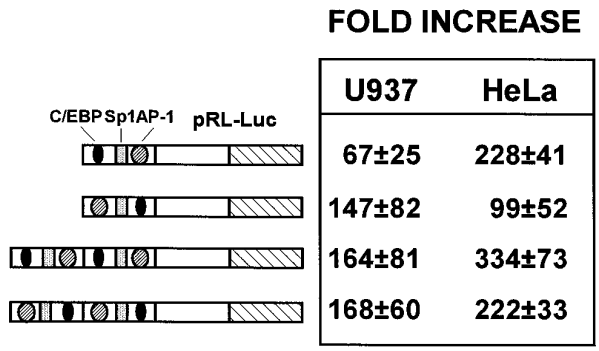

FIG. 8. **The −90/−50 fragment from the CD11c promoter (CESpAP sequence) enhances the activity of a heterologous promoter.** U937 and HeLa cells were transfected with the indicated prolactin promoter/luciferase-based reporter constructs and the luciferase activity determined and expressed relative to the activity produced by the prolactin promoter in the absence of any upstream sequence. Each construct was transfected at least three times using distinct DNA preparations. The average and standard deviations of three to four independent experiments are shown.

and Sp1–70 and Sp1–120 occupancy is completely independent on the presence or integrity of the CEBP-80 element (Ref. 23 and this paper). Moreover, anti-Sp1 antiserum or Sp1 consensus binding sites have no effect on the ability of C/EBPα to bind to oligonucleotides including both CEBP-80 and Sp1–70 (data not shown), further demonstrating that C/EBP proteins bind to CEBP-80 independently of adjacent sequence elements, although it is possible that Sp1 might facilitate the C/EBP transcriptional activity on the CD11c promoter and contribute to the distinct transactivation potential of C/EBPα, C/EBPβ, and C/EBPδ. The functional cooperation between C/EBPα and Sp1 on the CD11c promoter is in agreement with previously published data showing that both C/EBPα and C/EBPβ synergize with Sp1 to elicit transcriptional activation on a high-affinity C/EBP site is present, while only C/EBPβ provides transcriptional cooperation on a cryptic C/EBP-binding site (35).

Undifferentiated proliferating myeloid cells abundantly express C/EBPα, while myeloid cell differentiation or activation causes a gradual decrease of C/EBPα and a concomitant induction of C/EBPβ and C/EBPδ (33, 36). All the studies so far reported indicate that C/EBPα might be more important at earlier stages of the myeloid differentiation pathway, while C/EBPβ (and C/EBPδ) would be more relevant in the functional activation of differentiated myeloid cells (33, 37). Our results demonstrate that C/EBPα is the predominant C/EBP factor affecting the activity of the CD11c promoter in undifferentiated U937 and other proliferating myeloid cells, where CEBP-80 disruption greatly affects the promoter activity. The changes in CEBP-80-bound proteins observed by EMSA (increasing presence of C/EBPβ, gradual loss of CEBP-80 retarded complexes) suggest that the contribution of the distinct C/EBP factors to the CD11c gene transcription varies along myeloid differentiation. In this regard, the loss of CEBP-80-bound species along U937 PMA-triggered myeloid differentiation might be explained if only C/EBPα-containing dimers were capable of recognizing CEBP-80 or, alternatively, by the induction of other members of the C/EBP family known to produce non-functional C/EBP dimers (*e.g.* CHOP 10/GADD153) (38, 39).

C/EBPα transactivation of the CD11c promoter was not only affected by mutations at Sp1-binding sites but also by disrupting the AP1–60 AP-1-binding element. This finding, together with the described interactions between AP-1 and Sp1 family members on AP1–60/Sp1–70 (24), indicates that multidirectional functional interactions take place among the transcription factors bound to the most proximal region of the CD11c promoter. Furthermore, since Sp1 activity appears to be controlled by the retinoblastoma gene product (pRB) (40, 41) and members of the C/EBP family also interact with pRB and pRB-like proteins (42, 43), this multidirectional cooperation might be governed by pRB, thus coupling the CD11c integrin gene expression to the proliferative state of the cell. In this case, the proximal regulatory region of the CD11c promoter spanning from −130 to −50 would confer responsivenes not only to differentiation agents and tissue-specific stimuli but also to proliferative signals. As a preliminary analysis, and to determine whether the transcriptional behavior of the −90/−50 fragment, we have evaluated the effects of the CESpAP sequence on a heterologous promoter and demonstrated that a composite element including CEBP-80, Sp1–70, and AP1–60 is capable of greatly enhancing the activity of the prolactin promoter regardless of its orientation. Therefore, the −90/−50 fragment constitutes a positive regulatory unit within the CD11c gene promoter.

The expression of the CD11c/CD18 integrin is greatly increased upon monocyte extravasation (Ref. 1 and references herein) and we have previously hypothesized that this effect could be mediated by an extracellular matrix (ECM)-responsive element within the regulatory regions of the CD11c gene (44). Analysis of adhesion-generated intracellular signals have demonstrated that ECM recognition by integrins enhances AP-1 transcriptional activity (45, 46) and revealed the importance of a C/EBP-binding site within the β-casein gene ECM-responsive enhancer (47). Therefore, the AP-1- and C/EBP-binding sites within the CD11c promoter could potentially serve as switches for modulation of the CD11c/CD18 integrin expression in response to the state of cellular adhesiveness and depending on the integrins engaged in ECM attachment. Consequently, we are currently determining not only whether the CESpAP sequence is a bona fide tissue-restricted enhancer, but is capacity of conferring ECM-responsiveness. Moreover, the CD11c promoter is responsive to several myeloid differentiation stimuli and the monocytic differentiation-responsiveness precisely maps to the CESpAP region (25, 26). The differentiation-associated changes in C/EBP and AP-1 protein levels and in the occupancy of C/EBP-80 (this paper) and AP1–60 (26) strongly suggest that the differentiation responsiveness of the CD11c promoter relies on the combined action of C/EBP and AP-1 factors, a situation that has also been recently proposed for the transcriptional induction of collagenase-1 during monocytic differentiation (48). Thus, considering the opposite changes in the levels of c-Fos and C/EBPα, it is tempting to speculate that CD11c gene transcription would shift from C/EBP-driven to AP-1-driven during monocytic differentiation: CD11c gene transcription might be mostly CEBP-80-dependent C/EBPα-driven in proliferating undifferentiated cells and the weight of the CEBP-80-dependent transcription would gradually decrease along differentiation, due to lower C/EBPα expression (33) and to increased C/EBPβ, and possibly GADD153, levels. Conversely, the contribution of the AP1–60 element would concomitantly rise along monocytic differentiation as a consequence of the increased expression of c-Fos (49, 50) and, in this manner, the CD11c promoter activity in differentiated myeloid cells would be predominantly AP1–60-dependent and AP-1-driven, in agreement with the greatly decreased differentiation-inducibility seen upon mutation of the AP1–60 site (25, 26). Furthermore, at the light of the combinatorial theory for tissue-specific expression (reviewed in Ref. 51), the pattern of expression of c-Fos and C/EBPα, together with the presence of functional C/EBP- and AP-1-binding sites within the CD11c promoter, might represent an essential parameter for the restricted expression of the CD11c/CD18 integrin.

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CCAAT-Enhancer-binding Proteins (C/EBP) Regulate the Tissue Specific Activity of the CD11c Integrin Gene Promoter Through Functional Interactions with Sp1 Proteins

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